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Flavodon ambrosius sp. nov., a basidiomycetous mycosymbiont of *Ambrosiodmus* ambrosia beetles

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Abstract — Pure fungal cultures recovered from mycangia and fungal gardens of three *Ambrosiodmus* species were identified as a basidiomycetous fungus within the *Polyporales*. Culture-independent molecular analysis of the mycangia indicated that this fungus is the dominant symbiont transported by these beetles, and molecular phylogenetic analyses placed this fungus in a lineage with *Flavodon flavus*. We describe *Flavodon ambrosius* as a new species based on its phylogenetic position in relation to *F. flavus*, its role as a mycosymbiont rather than a free-living saprophyte, and its mode of asexual reproduction via arthroconidia.

Key words — *Basidiomycota*, nuclear ITS rDNA, 28S rDNA

Introduction

Flavodon Ryvarden (Ryvarden 1973) was described as a monotypic genus to accommodate *Irpex flavus* Klotzsch (Klotzsch 1833), whose classification had previously been questioned by Maas Geesteranus (1967). *Flavodon flavus* (Klotzsch) Ryvarden is a resupinate to conchate species of white-rot fungus in *Polyporales* with a hymenium that is dominantly poroid in resupinate forms but ages to hydnoid and which produces slightly ellipsoid, hyaline spores. The species is distinguished from related taxa in its yellow color that reacts to KOH by turning brown (Maas Geesteranus 1967). The hyphal system is dimitic, with thin generative and thickened skeletal hyphae possessing simple septa (Maas Geesteranus 1967) but otherwise morphologically unadorned at the microscopic level. Corner (1987), however, proposed monomitic *Polyporus cervinogilvus* Jungh. as "*Flavodon cervinogilvus*" (Jungh.) Corner (an invalid

name, lacking a basionym reference), thus opening interpretation of *Flavodon* to include monomitic and dimitic hyphal systems.

Flavodon is not uncommon in southern Asia from Pakistan to the Philippines (Maas Geesteranus 1967, Ryvarden & Johansen 1980, Corner 1987), tropical Africa to South Africa (Maas Geesteranus 1967, Ryvarden & Johansen 1980), and Australia (Ryvarden & Johansen 1980). *Irpex flavus* (the type species of *Flavodon*) was described from a North American specimen (Klotzsch 1833), but Ryvarden (1973) speculated that this locality might be a mislabeling error, perhaps from Mauritius (Indian Ocean). Gilbertson & Adaskaveg (1993) provided the first record of "*F. cervinogilvus*" in the United States from trees on the eastern coast of the Island of Hawai'i, and since then Miettinen et al. (2012) molecularly characterized a culture of *F. flavus* from Jamaica and placed the species alongside *Irpex lacteus* (Fr.) Fr. (Fries 1828), which is in the phlebioid clade of the *Polyporales* (Binder et al. 2013).

As part of an investigation into the mycosymbionts of *Ambrosiodmus* ambrosia beetles (*Coleoptera*, *Curculionidae*, *Scolytinae*, *Xyleborini*) in Florida, USA, Li et al. (2015) isolated fungi from mycangia of *A. lecontei* Hopkins, *A. minor* (Stebbing), and *A. rubricollis* (Eichhoff) from both stages of the ambrosia fungal life cycle: from the beetle mycangia as well as from their fungal gardens. To identify the fungi, Li et al. (2015) obtained ribosomal DNA (rDNA) sequences from both cultured isolates and culture-independent analyses of beetle mycangia, and these rDNA sequences identified the beetle mycosymbiont that dominated the mycangial fungal community as a relative of *Flavodon flavus*, as characterized by Miettinen et al. (2012). Additionally, microscopic examination of the fungus in culture and in situ in mycangia (Li et al. 2015) revealed hyphae with simple septa similar to those of the *Flavodon* type species, *F. flavus*, but no reproductive structures were observed.

Molecular and microscopic analyses indicated that this new fungus is a stable symbiont with *Ambrosiodmus* species and is horizontally transported from one generation to the next to act as a nutrient source via growth in the beetles' natal galleries in woody substrates. However, the two known *Flavodon* species have been observed only as saprophytic and free-living. Our new fungus, the first basidiomycetous mutualistic symbiont of ambrosia beetles, is proposed here as *Flavodon ambrosius*.

Materials & methods

Morphology of fungal cultures

Culture Hulcr 6853 from *Ambrosiodmus minor* was selected from analysis of Li et al. (2015) for morphological examination and typification. Subcultures were inoculated onto fresh BD Difco™ potato dextrose agar (PDA) medium, which was strengthened with

additional agar (5 g/L). Petri dishes (100 × 15 mm) of inoculated media were incubated at 25 °C until biomass had extended from the inoculation site to the edge of the dish (~4–5 d). Hyphae and arthroconidia were aseptically removed from the agar dishes, examined on glass slides, and photographed with an Olympus BX53F microscope equipped with phase optics and a ProgRes® SpeedXT core 3 camera (Jenoptik Optical Systems) using ProgRes® CapturePro 2.8.8. Aseptate arthroconidia (n = 20) were measured from which means (± standard deviations) were calculated.

Molecular phylogenetic analyses

We selected 28S rDNA sequences from Miettinen et al. (2012) that corresponded to the "*Byssomerulius* family", which included *Flavodon flavus* and related taxa. We aligned these sequences with those from cultured and culture-independent *Ambrosiodmus*-associated fungi (Li et al. 2015) using default settings in Geneious version 7.1.8 (http://www.geneious.com, Kearse et al. 2012). We used the Akaike information criterion (AIC) in jModeltest 0.1.1 (Guindon & Gascuel 2003, Posada 2008) to select the appropriate nucleotide substitution model. We conducted a maximum likelihood (ML) phylogenetic analysis with the model parameters in GARLI 2.01 (Zwickl 2006) to determine the best tree topology. We calculated bootstrap support values in GARLI from 100 search replicates, which we summarized with SumTrees (Sukumaran & Holder 2010). We conducted Bayesian phylogenetic analysis with the same parameters in MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003), in which two runs of four chains each were executed simultaneously for 1M generations, sampling every 100 generations. We summarized 7501 trees retained after a burn-in of 2500 trees in SumTrees to compute Bayesian posterior probabilities (BPP).

Taxonomy

Flavodon ambrosius D.R. Simmons, You Li, C.C. Bateman & Hulcr, **sp. nov.** Fig. 1

MycoBank MB 813875

Differs from other *Flavodon* species by participating in a mutualistic symbiosis with ambrosia beetles and by the formation of arthroconidia from vegetative hyphae in pure culture on agar medium.

Type — USA, Florida, Alachua County, Gainesville, 29°37′16″N 82°22′32″W), extracted from preoral mycangia of *Ambrosiodmus minor* caught by light-trapping, 12 Nov 2014, Hulcr 6853 (**Holotype**, BPI 893213; ex-type culture, ATCC TSD-21; GenBank KR119072, KR119075, KR119078).

Etymology — *ambrosius* (Lat.): referring to the symbiotic relationship of this fungus with an ambrosia beetle genus.

Colonies on PDA white, yellowing with age; mycelium along surface of agar projected aerially, cottony, irregular at margins; hyphae monomitic with simple septa, hyaline, smooth, with narrow hyphae (3–4.5 µm wide) branching from wide hyphae (7–8 µm wide). Arthroconidia hyaline; cylindrical, aseptate (but may appear septate due to incomplete fragmentation), either (2.9–) 5.1–9.5(–12.7) µm long (produced by narrow hyphae) or (4.4–)5.2–8.4(–10.2)

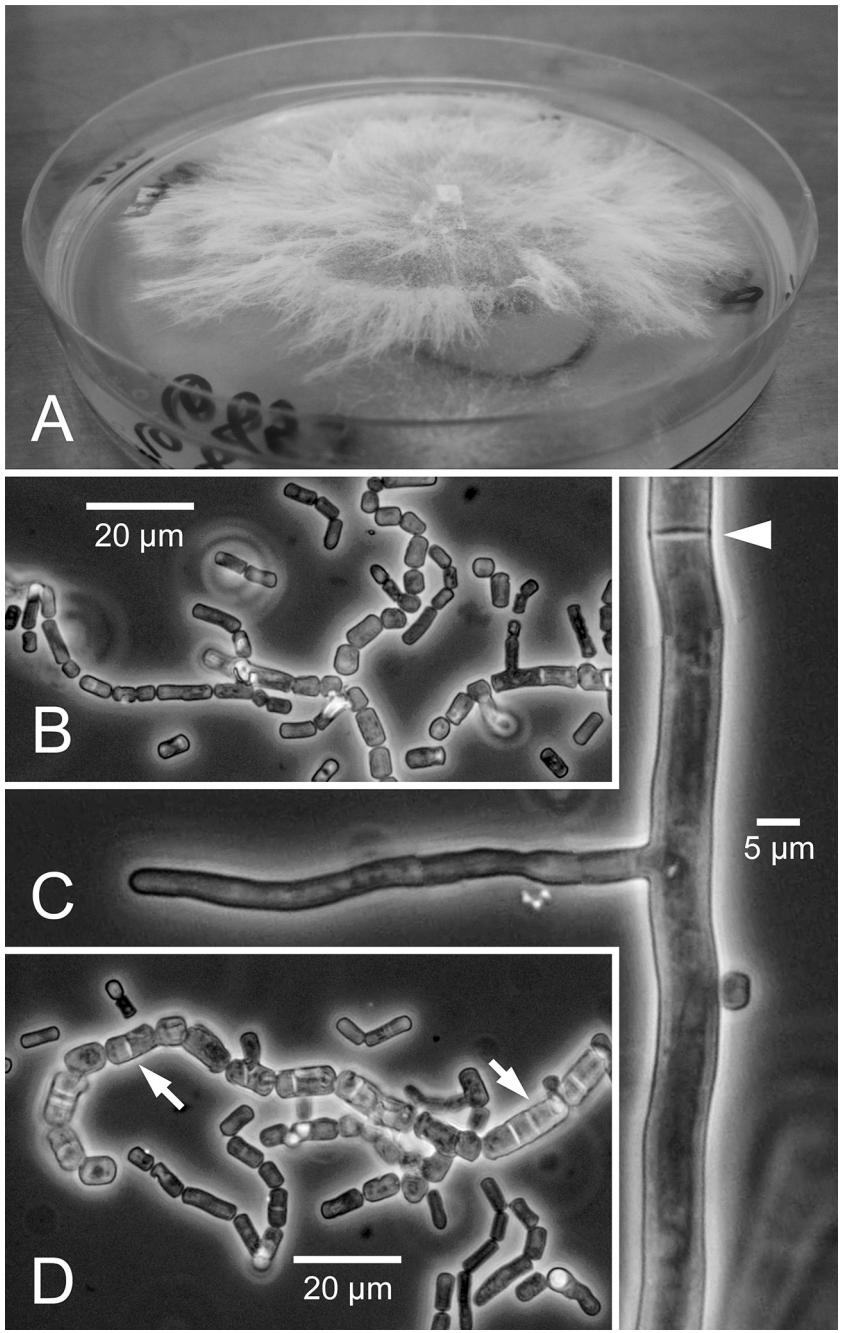
μm long (produced by wide hyphae). Sexual morph not observed. Associated with ambrosia beetles of tribe *Xyleborini*.

Additional specimens examined for sequence data — **USA**, **Florida**, Alachua County, Gainesville, 29°37′16″N 82°22′32″W, extracted from preoral mycangia of *Ambrosiodmus lecontei* inhabiting moribund wood of *Myrica cerifera*, 12 Nov 2014, Hulcr 6860 (GenBank KR119074, KR119077, KR119080); Hulcr 6855 (GenBank KR119073, KR119076, KR119079); extracted from preoral mycangia of *A. lecontei* inhabiting moribund wood of *Myrica cerifera*, 1 May 2015, Hulcr 7324 (GenBank KR871005); from fungal garden of *A. minor* in *Platanus occidentalis*, 30 Apr 2015, Hulcr 7346 (GenBank KR871006); extracted from preoral mycangia of *A. rubricollis* inhabiting moribund wood of *Liquidambar styraciflua*, 1 May 2015, Hulcr 7353 (GenBank KR871007); Hulcr 7354 (GenBank KR871008); from fungal garden of *A. rubricollis* in *Liquidambar styraciflua*, 1 May 2015, Hulcr 7373 (GenBank KR871009).

Discussion

Li et al. (2015) described the hyphal system of *Flavodon ambrosius* as dimitic based on hyphal size variation, leading to the assumption that narrow hyphae represented generative mycelial tissue and wider hyphae represented skeletal mycelial tissue. However, true thick-walled skeletal hyphae were not observed by Li et al. (2015) nor in this study, and our observations of arthroconidial production from both hyphal size classes would classify the mycelium of *F. ambrosius* as generative and therefore monomitic. With the description of "*F. cervinogilvus*" (Corner 1987), *Flavodon* was opened to the inclusion of monomitic taxa (although Ryvarden (1973) placed *Polyporus cervinogilvus* in *Oxyporus*). Another monomitic species previously placed in *Oxyporus* is *Emmia latemarginata* (Durieu & Mont.) Zmitr. et al. (Zmitrovich et al. 2006), which also produces arthroconidia (Stalpers 1978). Molecular phylogenies of nuclear ITS and 28S rDNA (Zmitrovich & Malysheva 2014) place *E. latemarginata* in a monophyly with *F. flavus*, although inclusion of this taxon in our 28S rDNA data set led to its placement in a polytomy with *F. flavus* and *I. lacteus*, with *F. flavus* on the shortest branch from the node (data not shown). *Emmia* Zmitr. et al. was described to accommodate *Polyporus latemarginatus* Durieu & Mont. [≡ *Oxyporus latemarginatus* (Durieu & Mont.) Donk (Donk 1966)] based primarily on its phylogenetic distance from other *Oxyporus* species, but we

Figure 1. *Flavodon ambrosius* on PDA medium grown at 25 °C (micrographs taken with phase contrast optics unless otherwise noted): A. cottony, white aerial mycelia above hyaline mycelia in agar radiating from inoculum plug at center of Petri dish (100 mm in diameter); B. dominant type of arthroconidia (<5 μm in diameter), produced from fragmentation of small hyphae; C. small hyphae extending laterally from large, supporting hyphae with simple septum (arrowhead) lacking clamp connection; D. chain of larger arthroconidia (>5 μm in diameter) produced from fragmentation of larger, supportive hyphae.


A
20 µm
B
5 µm
C
D
20 µm

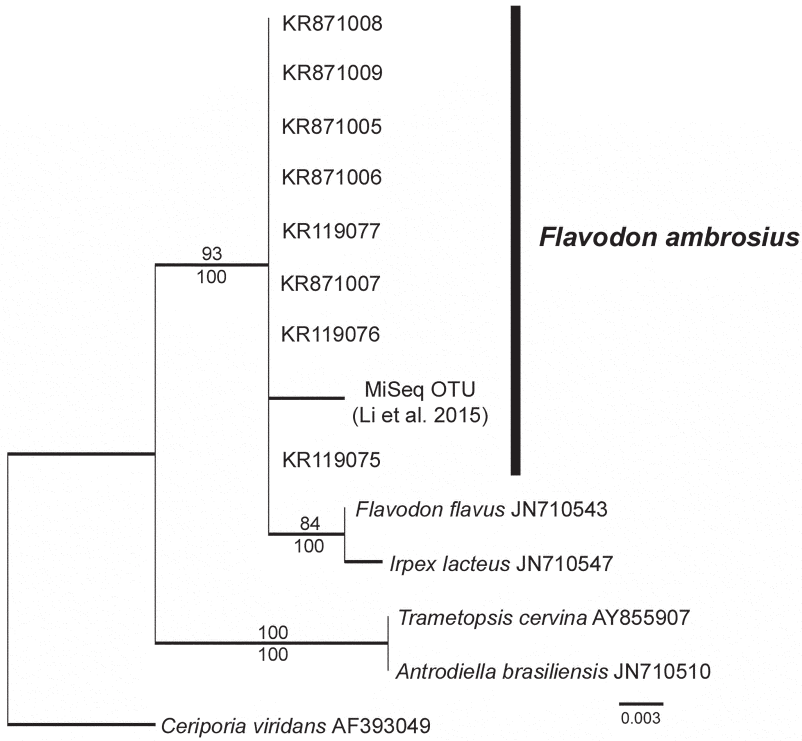


Figure 2. Best ML tree from GARLI analysis of 28S rDNA dataset of *Flavodon* and associated taxa, with binomials and GenBank accession numbers; trees from analyses were rooted with *Ceriporia viridans* sequence data. Values above branches represent ML bootstrap percentages >70% for that node from a summary of 100 replicates, and values below branches represent BPP for that node.

believe this species, along with the arthroconidia-producing *F. ambrosius*, is more appropriately placed in the molecularly defined, morphologically diverse *Flavodon*, which has priority over *Emmia*. For these reasons we describe our new taxon, *F. ambrosius*, in *Flavodon*, as suggested by Li et al. (2015).

Our 28S rDNA phylogeny (Fig. 2) places *Flavodon ambrosius* in a paraphyletic group with *F. flavus*. In their phylogenetic analysis of *Polyporales,* which included data from ribosomal and protein-coding DNA, Miettinen et al. (2012) placed *Flavodon flavus* in a lineage with *Irpex lacteus.* The authors contended that their evidence supported the separation of *I. lacteus* and

certain morphologically similar taxa, but they did not specifically address *F. flavus*. Indeed, their morphological generalization of *I. lacteus* (i.e., hymenium irregularly poroid when young and lacking clamp connections at septa) was very similar to that of *F. flavus*. However, the nuclear ITS rDNA sequences obtained from *F. ambrosius* better match *F. flavus* (90–91%) over *I. lacteus* (~88%) as both are presented by Miettinen et al. (2012). Therefore, we are confident in our placement of this new species in *Flavodon* until such time that more evidence justifies its reclassification within a different genus, either described or new.

Li et al. (2015) used Illumina MiSeq® culture-independent sequencing of 28S rDNA to assess the fungal community in *Ambrosiodmus minor* mycangia, while fungal isolations were made at the same locality and time. Li et al. (2015) found a single dominant operational taxonomic unit (OTU) comprising 94% of the resulting sequence reads after the removal of non-fungal and low frequency (<1%) reads. A BLAST query of the dominant OTU in GenBank produced 26 accessions with 99% identity similarity, indicative of the difficulty in differentiating polyporalean taxa using universal molecular markers (Binder et al. 2013). In particular, two *Flavodon flavus* accessions, including JN710543 (Miettinen et al. 2012) used by Li et al. (2015) for phylogenetic analyses, were among this set of 99% similar taxa. We included the MiSeq OTU in our phylogeny (Fig. 2), and it was placed in a polytomy with all other *F. ambrosius* isolates, even though it was on a longer branch, reflecting two polymorphic nucleotides. We conclude that the cultured *F. ambrosius* isolates from the fungus gardens in wood as well as from *Ambrosiodmus* mycangia represent the same species as the dominant OTU in *A. minor* mycangia detected by community meta-barcoding.

The variation of hyphal diameter between the mycangial fungi observed by Li et al. (2015) in *Ambrosiodmus lecontei* and cultured *F. ambrosius* may be explained by differing nutrient sources (the limited habitats selected by the beetle vs. the rich PDA medium) and by desiccation and processing during the fixation of the beetle heads for histological observations. Pure *F. ambrosius* cultures produced aerial hyphae, from which the arthroconidia were derived, that have similar dimensions to the fragmented reproductive propagules ("oidia") described by Tagaki (1967) from *A. rubricollis* galleries. Whether Tagaki observed *F. ambrosius* associated with native *A. rubricollis* remains a topic of investigation, and molecular analyses of pure fungal cultures from Japanese *A. rubricollis* individuals and galleries could indicate an international dispersal of *F. ambrosius*.

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